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Short-term and long-term effects of fatty acids in rat hepatoma AS-30D cells: The way to apoptosis

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Abstract

Arachidonic acid and, to a smaller extent, oleic acid at micromolar concentrations decreased the mitochondrial membrane potential within AS-30D rat hepatoma cells cultivated in vitro and increased cell respiration. The uncoupling effect of both fatty acids on cell respiration was partly prevented by cyclosporin A, blocker of the mitochondrial permeability transition pore. Arachidonic acid increased the rate of reactive oxygen species (ROS) production, while oleic acid decreased it. Both fatty acids induced apoptotic cell death of AS-30D cells, accompanied by the release of cytochrome *c* from mitochondria to the cytosol, activation of caspase-3 and association of proapoptotic Bax protein with mitochondria; arachidonic acid being a more potent inducer than oleic acid. Trolox, a potent antioxidant, prevented ROS increase induced by arachidonic acid and protected the cells against apoptosis produced by this fatty acid. It is concluded that arachidonic and oleic acids induce apoptosis of AS-30D hepatoma cells by the mitochondrial pathway but differ in the mechanism of their action: Arachidonic acid induces apoptosis mainly by stimulating ROS production, whereas oleic acid may contribute to programmed cell death by activation of the mitochondrial permeability transition pore.

Keywords: Fatty acid; Arachidonic acid; Oleic acid; Apoptosis; Hepatoma; Mitochondria; Reactive oxygen species (ROS); Respiration; Membrane potential

1. Introduction

Effects of nonesterified long-chain fatty acids on isolated mitochondria are well recognized (for reviews, see [1,2]). They mainly include an increase in the permeability of the inner mitochondrial membrane to protons that leads to uncoupling of oxidative phosphorylation. The protonophoric mechanism of action has been clarified as due to the transfer of the fatty acid anion by the mitochondrial adenine nucleotide transporter [3] and some other anion carriers of the inner mitochondrial membrane [4,5], accompanied by a spontaneous flip-flop movement of the undissociated fatty acid molecule. Independently of this carrier/flip-flop-mediated mechanism, a direct passage of H^+ through the inner mitochondrial membrane due to the opening, by fatty acids, of an unspecific pore, the so called permeability transition pore (PTP) [6], has been proposed [7–9]. Other effects of nonesterified long-chain fatty acids on isolated mitochondria include partial inhibition of respiration [10], increase of the permeability to monovalent and divalent metal cations, large-amplitude swelling (all reviewed in [1,2]) and modulation (inhibition [11] or stimulation [10]) of oxygen free radical production.

In contrast to these well-recognized and widely described effects on isolated mitochondria, much less is known on the influence of fatty acids on mitochondria within intact cells or perfused organs and the available information is often ambiguous and sometimes conflicting. For example, Soboll et al. [12–14] have shown that nonesterified fatty acids added to the perfusing fluid increase respiration of liver

Abbreviations: Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; $DCFH_2$-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; PBS, phosphate-buffered saline; PTP, permeability transition pore; ROS, reactive oxygen species; UV, ultraviolet light; $\Delta\psi$, mitochondrial transmembrane potential

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cells concomitantly with decreasing their ATP content and partial dissipation of the mitochondrial protonmotive force. On the other hand, other authors found that, under specific conditions, the stimulation by fatty acids of oxygen uptake in perfused liver [15] and isolated hepatocytes [16,17] was prevented by oligomycin, thus arguing against it being due to uncoupling. Such argument was also supported by Brand and co-workers [18,19] and ourselves [20] who found an increase, rather than a decrease, of the mitochondrial transmembrane potential ($\Delta\psi$) and redox state in hepatocytes treated with low concentrations of fatty acids. A more systematic study on the effect of various long-chain fatty acids on isolated cells have been carried out by Bernardi et al. [21–23] using a minimal deviation rat hepatoma MH1C1 (Morris hepatoma). They showed that unsaturated fatty acids, but not saturated ones, produced a decrease of $\Delta\psi$ that could be partially abolished by inhibitor of the permeability transition pore cyclosporin A.

Conflicting results concern the effect of fatty acids on production of reactive oxygen species (ROS) by cultured cells. While some authors found a decrease of ROS generation [24], some others observed a substantial stimulation [25,26].

Studies on long-term effects of fatty acids on cells mainly concern cell survival. Induction of cell death by nonesterified long-chain fatty acids, both saturated and unsaturated, is known since the pioneering work by Wenzel and Hale [27,28] on cultured cardiomyocytes and endothelioid cells. This has been confirmed by more recent studies on various cell types (reviewed in [2]). In these studies, cell damage and eventual death ensued several hours following supplementation of the culture medium with fatty acids and were not due to the detergent effect. There are good reasons to assume that in most of these cases cell death followed the apoptotic (programmed) rather than the necrotic pathway [23,29]. However, the mechanism(s) by which fatty acids elicit programmed cell death are not fully recognized. The possible agents that may been considered are disruption of cell energetics due to mitochondrial uncoupling and promotion of PTP opening.

The present investigation is an attempt to study in more detail the effects of selected nonesterified long-chain fatty acids on mitochondria within live cells with the aim to elucidate the mechanism that eventually lead to cell death. The object of this study is a highly malignant and poorly differentiated rat hepatoma AS-30D. This cell line has been chosen because it grows rapidly, can be cultivated in suspension (A. Villalobo, personal communication) and is easy to handle. AS-30D is derived from a solid liver tumour induced by feeding rats with the carcinogen 3′-methyl-4-dimethylaminoazobenzene and then cultivated in the ascites form in the peritoneal cavity of rats [30]. This cell line exhibits high glycolysis and, at the same time, high respiration rate and ATP production by oxidative phosphorylation [31–33]. In the present investigation, the cells were grown as cell culture in artificial medium, yielding very pure and relatively uniform cell population not contaminated with erythrocytes and other cells, as is usually the case when AS-30D is cultivated in the peritoneal cavity.

2. Materials and methods

2.1. Cell culture and treatment

AS-30D cells, kindly provided by Dr. Antonio Villalobo (Institute for Biomedical Research, National Research Council and Autonomous University of Madrid, Spain), were cultivated in RPMI-1640 medium (for detailed composition see www.irvinesci.com) containing 20 mM HEPES–NaOH (pH 7.4) and supplemented with 2 mM L-glutamine, 10% foetal calf serum and gentamycin 40 μg/ml at 37 °C in a humidified atmosphere of 5% CO_2 in air, essentially as recommended by A. Villalobo (personal communication). The cells were seeded in the fresh medium every 2–3 days after reaching a density of 1×10^6 cells/ml. The cells used for experiments have always been transferred to the fresh medium the day before. They were then collected by low-speed centrifugation at 150×*g* for 3 min at room temperature and suspended in PBS supplemented with 2 mM L-glutamine and gentamycin 40 μg/ml at a density of 5×10^6 cells/ml corresponding approximately to 1 mg protein/ml.

For long-term effects, fatty acids were added to such cell suspension in PBS and the mixture was kept at 37 °C for 30 min. Then, a four-fold volume of RPMI-1640 medium containing 10% foetal calf serum and other additions was added and the cells were cultivated under standard conditions (see above).

2.2. Oxygen uptake

Oxygen uptake by the cells was measured at 37 °C in PBS supplemented with 2 mM L-glutamine and 5 mM Na-pyruvate using a Clark-type oxygen electrode (YSI, Yellow Springs, OH) equipped with an electronic device to plot the first derivative of the oxygen concentration trace (equivalent to the O_2 uptake rate).

2.3. Flow cytometry

This was performed using a FACSCalibur instrument (Becton-Dickinson, Warsaw, Poland) equipped with an argon laser (488 nm excitation) and using CellQuest software. The photomultipliers used will be specified for the individual procedures (see below).

2.4. Mitochondrial membrane potential

This was measured using flow cytometry of cells stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) [34]. The cells were harvested, suspended in PBS supplemented with L-glutamine at a density of at least 2×10^6/ml and incubated for 15 min in the dark at room temperature with 6 μM JC-1. After washing twice with PBS and resuspending in the same solution, the cells were immediately analysed using flow cytometry with photomultipliers sensitive to green (FL1) and orange red (FL2) light.

2.5. Production of reactive oxygen species

ROS production within intact cells was followed by measuring formation of H_2O_2 using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). The cells, suspended in 1 ml PBS supplemented with L-glutamine, were incubated with 20 μM $DCFH_2$-DA for 30 min at 37 °C and thereafter analysed by flow cytometry [35] using green light-sensitive photomultiplier (FL1).

Alternatively, H_2O_2 production was measured fluorimetrically with the same probe at the excitation and emission wavelengths of 500 and 520 nm, respectively, in Shimadzu model RF-5000 spectrofluorimeter.

2.6. Determination of cytosolic Ca^{2+} concentration

This was accomplished using calcium fluorescent dye Fura-2. The cells were loaded with the probe by incubation with the acetoxymethyl ester of Fura-2 (1 μg/ml) in the full culture medium for 15 min at 37 °C. Then, the cells were washed with a medium containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM phosphate, 25 mM HEPES (pH 7.2), 5 mM glucose, 1 mM pyruvate and 0.1

mM $CaCl_2$ and examined in the same medium in which $CaCl_2$ was replaced by 50 μM EGTA. Fluorescence was recorded in the ratio mode at 340/380 nm excitation and 510 nm emission wavelengths [36].

2.7. Lipid extraction and separation

The cells incubated with [^{3}H]oleic acid were sedimented by brief centrifugation, extracted with a mixture of chloroform and methanol and the concentrated chloroform extracts were subjected to thin-layer chromatography on silica plates as described previously [37]. Spots of fatty acids, phospholipids and mono-, di- and triglycerides were visualized with iodine vapour and scrapped for scintillation counting. The cell-free supernatant was deproteinized and both the sediment (consisting mainly of serum albumin) and the soluble fraction were counted.

2.8. Caspase-3 activity

This was measured with the synthetic substrate Ac-DEVD-pNA as described previously [38]. The results were normalized for the amount of the cells as based on lactate dehydrogenase activity [39].

2.9. Confocal microscopy

The cells were incubated with 100 nM MitoTracker CMXRos in the dark at 37 °C for 10 min, briefly rinsed with the medium and fixed with 4% paraformaldehyde for 15 min. After washing off paraformaldehyde, the cells were incubated for 1 h with primary polyclonal anti-bax antibody in the presence of 0.2% saponin, followed by staining with appropriate fluorescently conjugated secondary antibody. The cells were viewed in a Leica TCS SP2 spectral confocal and multiphoton microscope using a 100× oil immersion objective.

2.10. Induction of apoptosis by ultraviolet light illumination

The cells were subjected to a pulse of UV light of 254 nm and energy output of 100 J/m^2 using a UV Stratalinker 2400 instrument (Stratagene, La Jolla, CA).

2.11. Estimation of apoptosis

A quantitative estimation of the number of cells undergoing apoptosis was performed by flow cytometry of cells stained with propidium iodide [40] as described [41], using orange-red light emission (FL2). In this procedure, the DNA content frequency histograms enabled to discriminate between cells with normal (diploid) DNA and those forming a broad hypodiploid DNA peak, the sub-G_1 population.

To discriminate between cells dying by apoptosis and those undergoing necrosis a combined staining with annexin V [42] and 7-aminoactinomycin [43] was performed. Annexin V used in this procedure was conjugated with phycoerythrin and the staining procedure followed the producer's protocol (BD PharMingen, San Diego, CA). Flow cytometry was recorded using photomultipliers sensitive to orange-red (FL2) and far red (FL3) light.

2.12. Cytochrome c release

For measuring leakage of cytochrome *c* from mitochondria to the cytosol, about 5×10^6 cells were subjected to a mild permeabilisation of the plasma membrane with digitonin as described previously [44,45], except that the concentration of digitonin was increased to 400 μg/ml. Cytochrome *c* in the supernatant was detected by immunoblotting using monoclonal anti-cytochrome *c* antibody.

2.13. Chemicals

Fatty acids, oligomycin, CCCP, FCCP and valinomycin were from Sigma (St. Louis, MO). Fatty acids were used as 10 mM stock solutions in dimethylsulphoxide. Solutions of arachidonic and oleic acids were kept tightly stoppered under nitrogen at −20 °C. CsA was from Novartis (Basel, Switzerland) or from the Institute of Biotechnology and Antibiotics (Warsaw, Poland). Ac-DEVD-pNA was from Calbiochem-Novabiochem (La Jolla, CA); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Oxis International Inc., Portland, OR); $DCFH_2$-DA, MitoTracker CMXRos, Fura-2 acetoxymethyl ester and JC-1 were from Molecular Probes (Eugene, OR). Annexin V conjugated with phycoerythrin, 7-aminoactinomycin and monoclonal anti-cytochrome *c* antibody were from BD PharMingen (San Diego, CA). Anti-bax polyclonal antibody was purchased from Becton-Dickinson (Warsaw, Poland). Other chemicals were of the highest purity grade commercially available. [9,10-^{3}H] Oleic acid was from Amersham (UK). RPMI-1640 medium was supplied by the Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland).

3. Results

3.1. Respiration

AS-30D cells, harvested after 24 h of cultivation in the fresh medium, exhibited a high rate of respiration, comparable to that of Ehrlich ascites tumour cells [45]. Oligomycin strongly decreased the respiration rate, whereas chemical protonophores, e.g., FCCP, were stimulatory (Fig. 1, trace A). This shows that in cultivated cells mitochondria were in an intermediate state between the resting state (state 4) and fully active state (state 3). This is similar to the situation in AS-30D cells cultivated in the peritoneal cavity of rats [33] and indicates that, under aerobic conditions, ATP utilized for the energy needs of the cell comes predominantly from oxidative phosphorylation. If the respiration of fully uncoupled cells was assumed as 100%, then the normal respiration was 63±7% and the resting state (+oligomycin) amounted to 21±4% (mean values for 5 experiments± S.D.; see legend to Fig. 1).

To study the uncoupling effect of fatty acids they were added to the cell suspension in small portions after oligomycin. As shown in Fig. 1 (traces B–E), these additions elicited stepwise increases of the respiration rate that, eventually, reached the level of fully uncoupled respiration. However, FCCP added after the last (fully uncoupling) addition of the fatty acid resulted in a substantial decrease of the respiration rate (Fig. 1, traces B and D). This picture was remarkably changed by CsA, blocker of the mitochondrial permeability transition pore. Firstly, CsA by itself produced a small decrease of the respiration rate (traces C and E). Secondly, it partly prevented the uncoupling effect of fatty acids. And thirdly, it prevented or significantly diminished the inhibitory effect of FCCP added following the fatty acid (compare traces C versus B, and E versus D). These effects can be interpreted in the sense that the uncoupling effect of arachidonic and oleic acids within AS-30D cells was partly due to PTP opening, similarly as already described for isolated mitochondria [7–9]. The mechanism of the inhibition by FCCP and its partial prevention by CsA is less clear.

3.2. Mitochondrial membrane potential

Application of flow cytometry to AS-30D cells stained with the mitochondrial membrane potential probe JC-1 revealed two distinct populations characterized by predominantly high and low $\Delta\psi$ values, respectively (Fig. 2A). Treatment of the cells with the potassium ionophore valinomycin plus the chemical

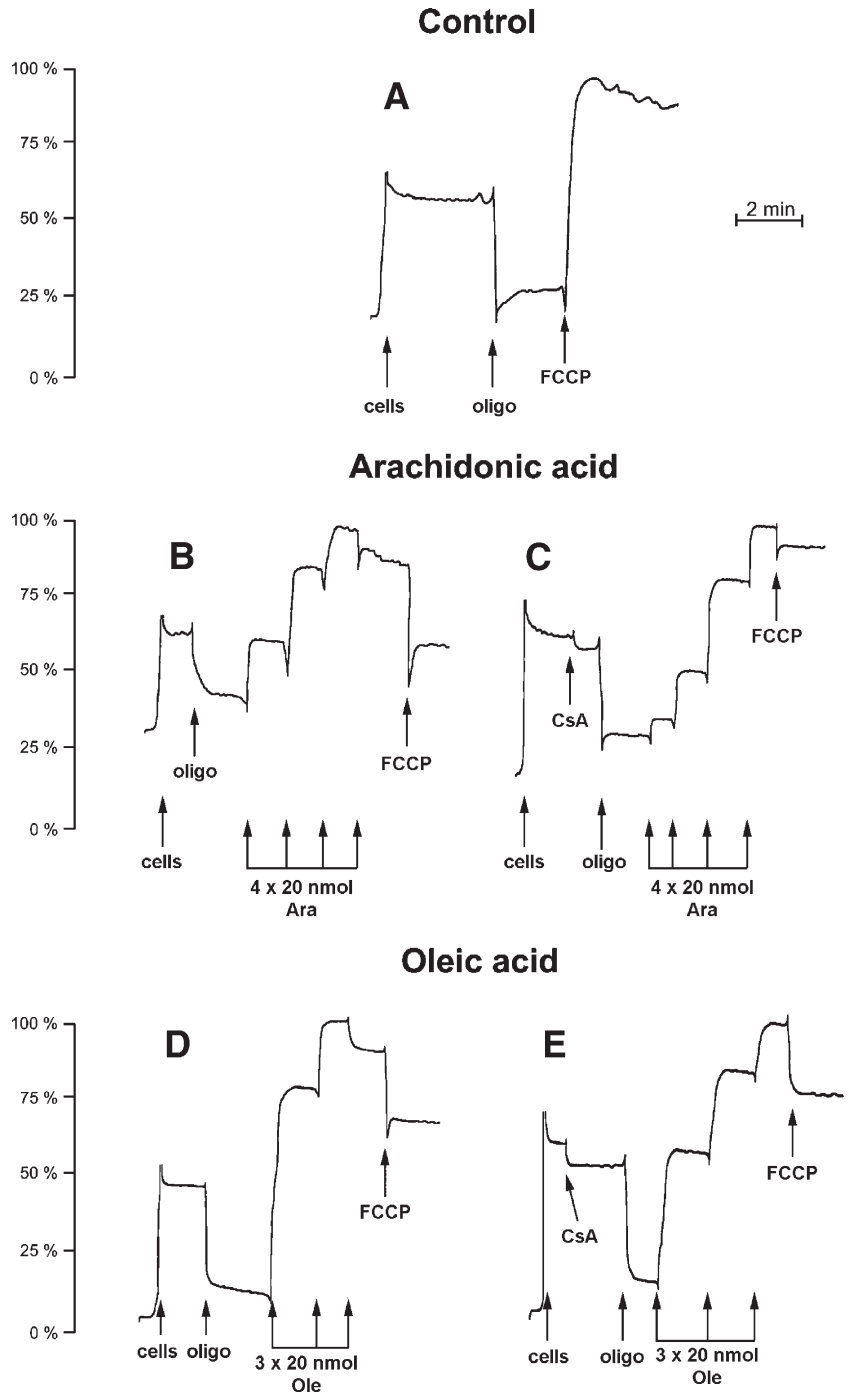

Fig. 1. Effect of arachidonic acid and oleic acid on the respiration of AS-30D cells. The total volume was 1.0 ml and the amount of the cells was about 5×10^6, corresponding to 1.0 mg protein. The traces show the rates of O_2 uptake in percentage of the maximal (fully uncoupled) respiration that amounted to 35±12 ng atom oxygen/min per mg protein (n=5). Additions: oligomycin (oligo), 1.7 μM; FCCP, 1 μM; CsA, 1.7 μM; arachidonic acid (Ara) and oleic acid (Ole), 20 nmol at each arrow. Traces in the same row (B and C, and D and E, respectively) are for the same cell preparations. Typical traces out of at least three similar are shown.

protonophore FCCP completely collapsed $\Delta\psi$ within intact cells and resulted in a shift of practically all the cells to the low $\Delta\psi$ population (Fig. 2B). Both arachidonic acid and oleic acid added to the cells suspended in PBS medium produced a collapse of $\Delta\psi$ within intact cells in a dose-dependent manner that could be observed within 30 min after addition of the acids (Fig. 2C–F), i.e., the time required to load the cells with JC-1 and run the measurement. There was no apparent effect of CsA on the proportion between high $\Delta\psi$ and low $\Delta\psi$ populations (not shown). It has, however, to be kept in mind that respiration experiments (Fig. 1) depict immediate effects of fatty acids, whereas $\Delta\psi$ measurements show the results after 30 min when half of the added fatty acid might have been metabolized (see Metabolism of added fatty acid, Chap. 3.5). Hence, the effect of CsA may be less visible in the latter case.

A different picture emerged in long-term experiments. As specified under Materials and methods, 30 min after addition of the fatty acid, the cell suspension in PBS was diluted with a

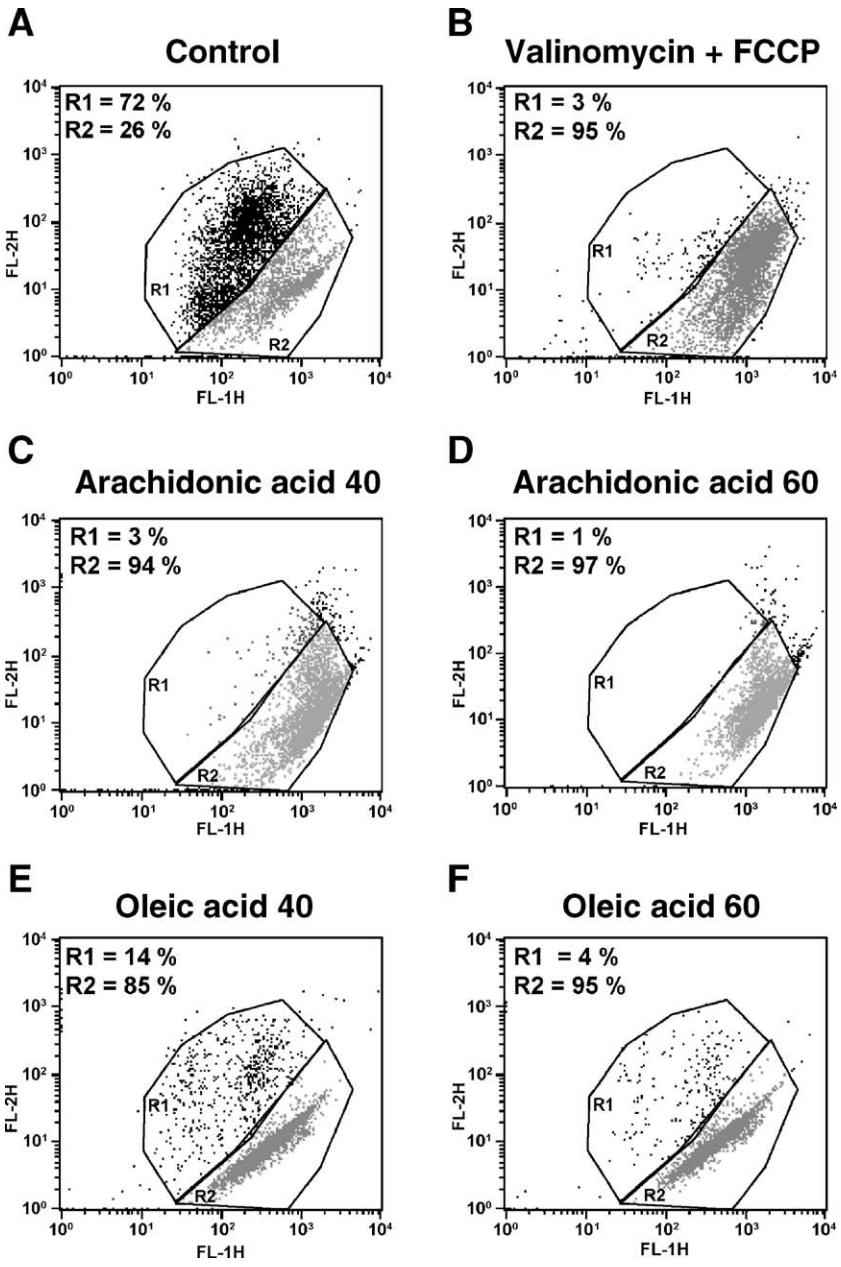

Fig. 2. Effect of arachidonic acid and oleic acid on mitochondrial membrane potential ($\Delta\psi$) within AS-30D cells. The cells, stained with JC-1, were analysed by flow cytometry 30 min after supplementation with the indicated concentration of the fatty acid (expressed in nmol/mg protein). Cells supplemented with 2 μM valinomycin plus 1.7 μM FCCP, that completely collapse $\Delta\psi$, are shown for comparison. The upper left hand area in each panel corresponds to cells with high $\Delta\psi$ (red fluorescence), and the lower right hand area to cells with low $\Delta\psi$ (green fluorescence). The numbers indicate the corresponding percentage distribution.

four-fold volume of the cultivation medium containing 10% foetal serum that contained serum albumin with its high fatty acid-binding capacity [46]. If the cells were stained with JC-1 2 h later, the effect of arachidonic acid on $\Delta\psi$ completely disappeared and the proportion of the cells with high and low $\Delta\psi$ returned to the same level as in control cells and was maintained for next 24 h (Table 1).

3.3. Generation of oxygen free radicals

Since the superoxide anion ($O_2^{\bullet -}$) formed intracellularly during one-electron reduction of O_2 is rapidly transformed to hydrogen peroxide due to high activity of superoxide dismutases, both mitochondrial (Mn-SOD) and cytosolic (Cu, Zn-SOD), determination of intracellular formation of H_2O_2 can be a good measure of ROS production. This was performed using the H_2O_2-sensitive probe $DCFH_2$. It was found that arachidonic acid applied at the concentration of 40 nmol/mg protein considerably increased the rate of ROS production by AS-30D cells (Fig. 3), whereas palmitic acid used at the same or higher concentration was ineffective and oleic acid resulted in a significant diminution of ROS production (Fig. 3). As expected [35,47], the protonophore CCCP lowered ROS production in control cells and also significantly decreased the potentiating

Table 1
Short-term and long-term effects of arachidonic acid on $\Delta\psi$

Time	10 min	2 h	4 h	6 h	24 h
high $\Delta\psi$/low $\Delta\psi$ ratio					
Control	1.9±0.4	1.6±0.4	1.4±0.2	1.5±0.2	1.4±0.2
With arachidonic acid	0.0	1.5±0.3	1.1±0.1	1.0±0.1	1.3±0.1

The cells (5×10^6 per ml) in PBS were supplemented with arachidonic acid, 60 nmol/mg protein. The first sample for $\Delta\psi$ measurement was taken after 10 min. Twenty min later the cell suspension was diluted with a four-fold volume of the cultivation medium containing 10% foetal serum and the samples for $\Delta\psi$ measurement were taken at the indicated times. The numbers (mean values for two experiments±range) represent the ratios between cell populations with high and low $\Delta\psi$, as determined by flow cytometry after JC-1 staining (see Fig. 2).

effect of arachidonic acid (Fig. 3). Thus, the effects of oleic acid could be ascribed to its uncoupling action, whereas that of arachidonic acid is evidently specific to that polyunsaturated fatty acid. The effect of arachidonic acid on ROS production could only be observed shortly after addition to the cell suspension and disappeared in long-term experiments already after 2 h when the cell suspension was diluted with the cultivation medium containing foetal serum (not shown).

Trolox, a water-soluble derivative of tocopherol with potent antioxidant properties [48,49], prevented arachidonic acid-induced ROS production (Table 2). Its effect on AS-30D apoptosis will be described further on (Chap. 3.6).

3.4. Cytosolic Ca^{2+} concentration

When the cells were supplemented with arachidonic acid, an increase of the cytosolic Ca^{2+} concentration could be observed. In nominally Ca^{2+}-free medium, this increase was small (Fig. 4A). However, in the presence of Ca^{2+} in the external medium, Ca^{2+} concentration in the cytosol was tripled, i.e., was increased by about 100 nM (Fig. 4B). This indicates that arachidonic acid both mobilizes calcium from its intracellular stores, presumably

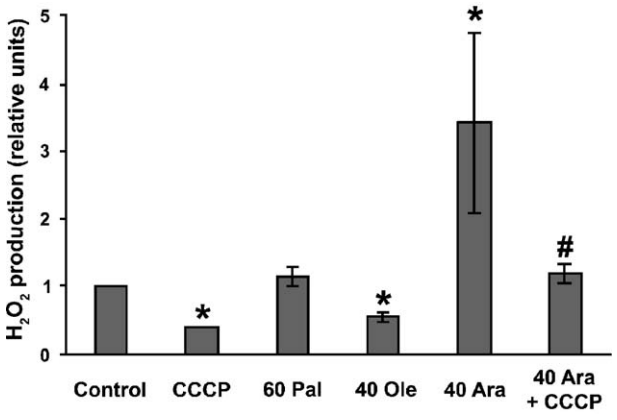

Fig. 3. Production of reactive oxygen species. The rate of ROS production, measured as H_2O_2 formation, was determined by flow cytometry with $DCFH_2$-DA. Palmitic acid (Pal), oleic acid (Ole) and arachidonic acid (Ara) were added at the amounts indicated (in nmol/mg protein) and CCCP at 5 μM final concentration. ROS formation for control cells was assumed as 1. Mean values± S.D. for 5 experiments are presented. Statistical significance: $^*P<0.05$ with respect to the control; $^{\#}P<0.05$ with respect to both CCCP alone and arachidonic acid alone.

Table 2
Reversal by Trolox of the increased ROS production and apoptosis induced by arachidonic acid

Additions	Rate of H_2O_2 production (relative)	Apoptosis (cells in sub-G_1 phase as % of total)
None	1	11±4
Trolox	0.7±0.2	13±3
Arachidonic acid	2.1±0.3[a]	40±7[c]
Trolox plus arachidonic acid	1.0±0.3[b]	11±2[d]

The cells (5×10^6 per ml) were suspended in PBS supplemented with 2 mM L-glutamine. Trolox, 2 mM, was added where indicated, followed 5 min later by arachidonic acid, 60 nmol/mg protein, also where indicated, and generation of ROS was measured by determining the rate of H_2O_2 production (see also legend to Fig. 3). Thirty minutes after these additions the medium was diluted by a four-fold volume of RPMI-1640 medium and incubated for 24 h. Apoptosis was then measured using propidium iodide staining (see Materials and methods). Mean values±S.D. for 3 experiments are shown. Statistical significance of the results in terms of Student's *t*-test was: [a]$P<0.05$ with respect to control (no additions); [b]$P<0.05$ with respect to arachidonic acid alone; [c]$P<0.01$ with respect to control; [d]$P<0.01$ with respect to arachidonic acid alone.

the endoplasmic reticulum, as well as increases Ca^{2+} influx from the medium.

3.5. Metabolism of added fatty acid

To investigate the fate of added fatty acid, the cells (5×10^6 cells/ml) suspended in PBS were supplemented with [^{3}H]oleic acid, 80 nmol/mg protein, equivalent to about 1 μCi. Thirty minutes later, the suspension was diluted with a four-fold volume of the cultivation medium containing 10% foetal calf serum (see Materials and methods) and cultivated up to 24 h. Aliquots were taken at various time intervals, centrifuged, and analysed for the distribution of the label between the cells and the medium and between various lipid species within the cells. It was found that, immediately after addition of oleic acid, about 60% of the label became bound to the cells and all of it was identified by thin layer chromatography as nonesterified fatty acid (Fig. 5). Within 30 min in PBS, about half of this label was gradually incorporated into phospholipids and di- and triglycerides. Then, after dilution of the cell suspension with the serum-containing medium most of the remaining nonesterified oleic acid passed to the medium, presumably bound to serum albumin, so that the amount still associated with the cells in the nonesterified form did not exceed 2% of the amount initially added (Fig. 5).

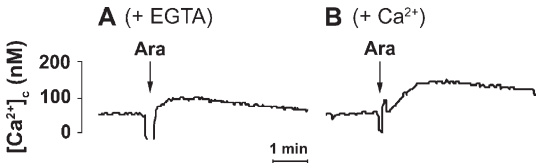

Fig. 4. Effect of arachidonic acid on the level of cytosolic Ca^{2+}. The cells loaded with Fura-2 (see Materials and methods) were incubated in a medium containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM phosphate, 25 mM HEPES (pH 7.2), 5 mM glucose, 1 mM pyruvate, 50 μM EGTA and oligomycin, 1 μg/ml. In trace B, the medium was additionally supplemented with 2 mM $CaCl_2$. Arachidonic acid (Ara) was added at the amount of 60 nmol/mg protein.

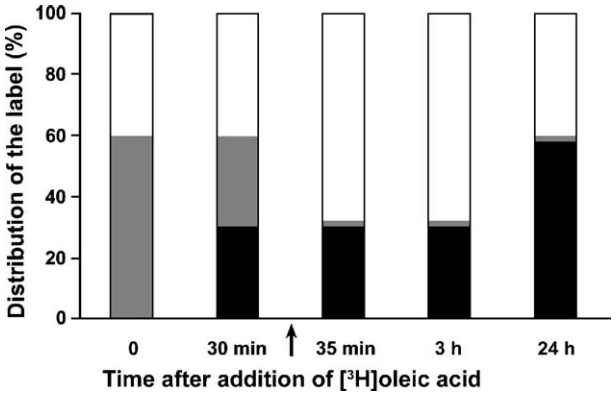

Fig. 5. Fate of oleic acid in AS-30D cells during incubation. To the cells suspended in PBS [^{3}H]oleic acid was added at the amount of 80 nmol/mg protein (corresponding to about 1 μCi) and the incubation was carried at 37 °C. After 30 min (arrow), the mixture was diluted with the complete incubation medium. Aliquots were withdrawn at the times indicated and analysed for cell-bound nonesterified fatty acid (gray columns), esterified fatty acid (mostly as phospholipids and di- and triglycerides; black columns) and radioactivity present in the extracellular medium (mostly nonesterified fatty acid, white columns). A schematic picture based on two experiments is shown.

3.6. Apoptosis

Quantitative evaluation of apoptosis by flow cytometry of propidium iodide-stained cells revealed an increasing proportion of the cells recorded in the sub-G_1 phase, characteristic for apoptosis [40], with increasing concentrations of arachidonic acid. Arachidonic acid applied at the concentration of 20 nmol/mg cell protein was without effect in this assay (not shown, although it already increased caspase-3 activity as will be shown in Chap. 3.7), but the concentration twice as high resulted in about 30% of apoptosis, whereas 60 nmol/mg protein produced apoptosis in 50% of the cells (Fig. 6). Further increasing the concentration of arachidonic acid resulted in a drastic decrease of the total number of the cells, probably due to necrosis and/or lysis (not shown). Oleic acid was much less effective than arachidonic acid at comparable concentrations, whereas palmitic acid was ineffective. UV irradiation, a known apoptogenic factor, applied for comparison, was highly effective (Fig. 6).

Another quantitative method of measuring apoptosis was flow cytometry of the cells stained with annexin V-phycoerythrin and 7-aminoactinomycin. In addition, this approach enabled a rough differentiation between cells undergoing apoptosis and necrosis. Cells that stained with annexin V-phycoerythrin but not with 7-aminoactinomycin were regarded as early apoptotic, whereas those stained with 7-aminoactinomycin only as necrotic. Staining with both probes indicated late apoptosis and/or secondary necrosis [42]. According to this convention treatment with 60 nmol arachidonic acid per mg protein resulted in early plus late apoptosis of about 50% of the cells (quadrants B+C in Fig. 7). Oleic acid was somewhat less effective (about 30% apoptosis). Again, UV irradiation resulted in a high degree of apoptotic cell death (Fig. 7).

The antioxidant Trolox completely prevented the proapototic action of arachidonic acid along with the protection against

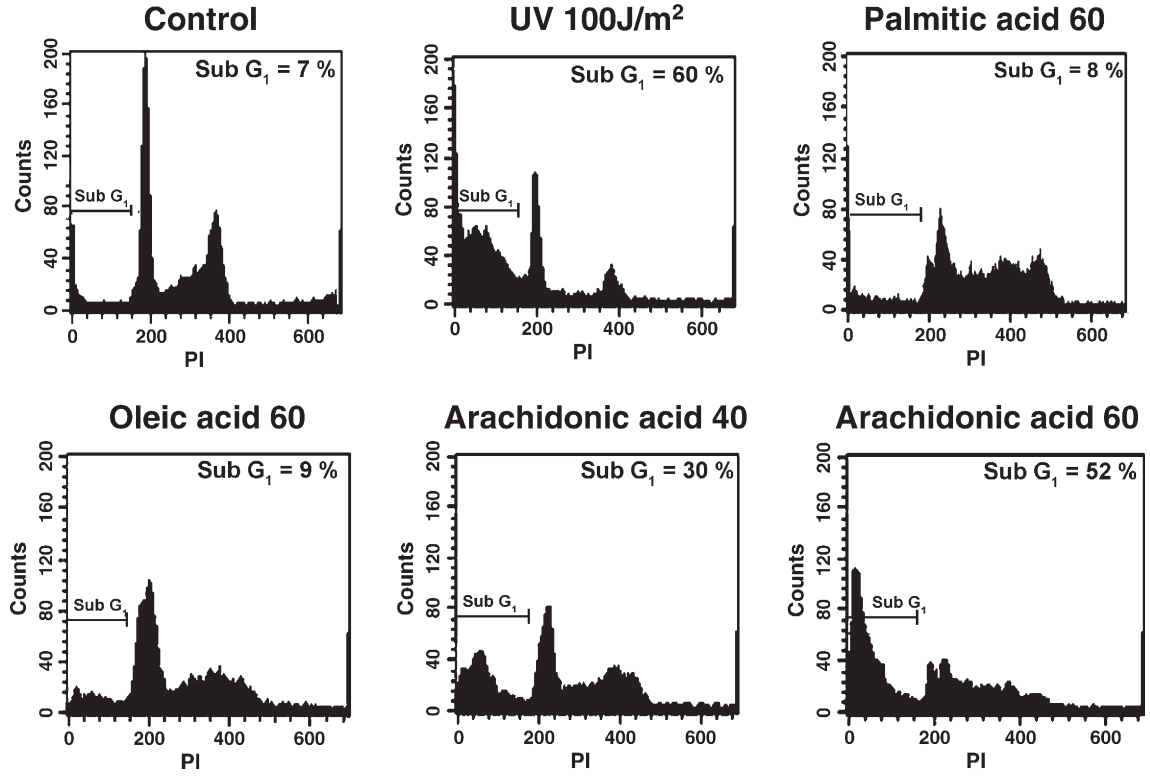

Fig. 6. Flow cytometry of AS-30D cells stained with propidium iodide. The cells were incubated with the indicated concentrations of the fatty acids (expressed in nmol/mg protein) for 24 h, or examined 24 h following UV irradiation. The sub-G_1 fraction, characteristic for apoptotic cells, is indicated by the horizontal bar and its percentage is shown in the upper right corner of each panel. A representative experiment is presented.

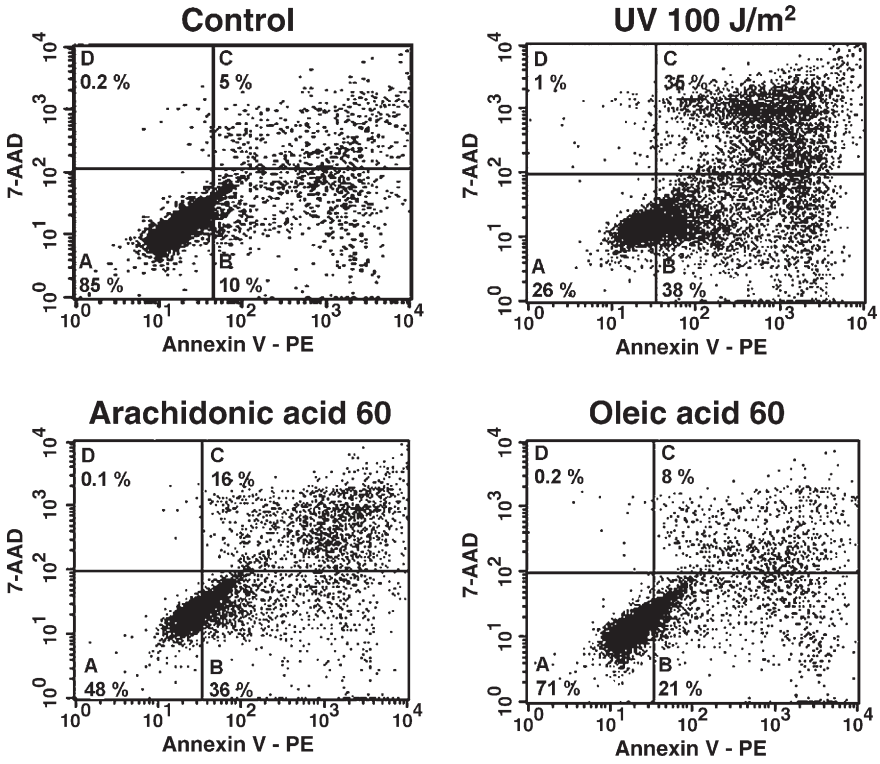

Fig. 7. Flow cytometric analysis of AS-30D cells stained with annexin V-phycoerythrin (Ann V-PE) and 7-aminoactinomycin (7-AAD) after incubation with oleic or arachidonic acid, 60 nmol/mg protein, for 24 h, or 24 h following UV irradiation. Control cells were incubated for the same time without fatty acids. Unchanged cells are located in quadrant A, early apoptotic cells (AnnV-PE positive) in quadrant B, late apoptotic cells (AnnV-PE/7-AAD double positive) in quadrant C and necrotic cells (7-AAD single positive) in quadrant D.

increased ROS production (Table 2). The chemical protonophore CCCP exhibited a small proapoptotic action by itself but if present in the incubation medium together with arachidonic or oleic acids had no additional effect (not shown).

In an attempt to investigate a possible protective effect of CsA on fatty acid-induced apoptosis we encountered a technical problem; namely, 5–10 μM CsA alone increased the proportion of apoptotic cells after 24 h by about 15%.

3.7. Biochemical changes accompanying apoptosis

Apoptosis is known to be accompanied by translocation of the proapoptotic protein Bax from the cytosol to mitochondria [50], release of cytochrome *c* from mitochondria to the cytosol [51,52] and activation of caspases [53,54].

To investigate the release of cytochrome *c* from mitochondria to the cytosol, the cells, 24 h after supplementation with the fatty acid, were made permeable with low concentration of digitonin, sufficient to permeabilise the plasma membrane but too low to affect the outer mitochondrial membrane, and leakage of cytochrome *c* to the external medium was examined by immunoblotting (for details, see Materials and methods). The results, illustrated in Fig. 8, show that arachidonic acid (60 nmol/mg protein) and UV irradiation resulted in a release of substantial amounts of cytochrome *c*, whereas the release of this haemoprotein by the same concentration of oleic acid was much smaller and that from control cells was negligible.

Caspase-3 activity was measured in the cell lysate 24 h after supplementation of the culture with fatty acids. As shown in Fig. 9, arachidonic acid produced a dose-dependent increase of the activity. Interestingly, the effect was manifested at the concentration of arachidonic acid as low as 20 nmol/mg protein that had almost no effect on apoptosis examined by flow cytometry. Arachidonic acid at a concentration of 60 nmol/mg protein resulted in tripling caspase-3 activity. Oleic acid had much lower effect, whereas UV irradiation activated the enzyme enormously.

Translocation of Bax from the cytosol to mitochondria was investigated using confocal microscopy. Mitochondria within the cells were stained with MitoTracker CMXRos and Bax was visualized with anti-bax antibody. In control cells, green fluorescence staining for Bax was diffuse

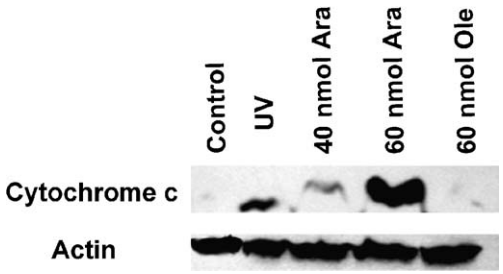

Fig. 8. Release of cytochrome *c* from mitochondria to the cytosol. The cells were incubated for 24 h without (control) or with the indicated concentrations of arachidonic or oleic acid or after UV irradiation (100 J/m^2).

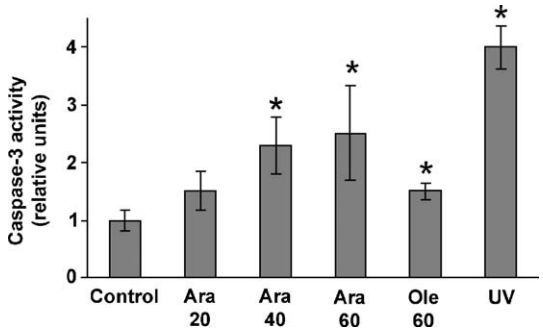

Fig. 9. Activation of caspase-3. The cells were incubated for 24 h without (control) or with the indicated concentrations of arachidonic or oleic acid (expressed in nmol/mg protein) or after UV irradiation (100 J/m^2). Caspase-3 activity for the control was assumed as 1 and the values were normalized on the basis of lactate dehydrogenase activity as marker for the cell cytosol. Mean values±S.D. for 4 experiments; statistical significance ($P<0.05$) is indicated with asterisk (*).

throughout the cytoplasm, whereas after treatment of the cells with arachidonic acid, 60 nmol/mg protein, Bax formed distinct punctated aggregates that could be assumed to represent mitochondrial localization (Fig. 10). In contrast, massive occluded mitochondria stained with MitoTracker could be observed in control cells, whereas the staining was much weaker and dispersed after arachidonic acid (Fig. 10). This change in mitochondrial staining could be due to mitochondrial fission that often accompanies apoptosis [55]. It may also result from a partial dissipation of $\Delta\psi$, since MitoTracker CMXRos is distributed between the cytosol and mitochondria according to the transmembrane potential and therefore its accumulation in mitochondria becomes reduced when $\Delta\psi$ decreases.

4. Discussion

The present results clearly indicate that apoptosis in AS-30D cells can be induced by unsaturated fatty acids, especially by arachidonic acid, with only a small or no effect of saturated fatty acids. Thus, AS-30D belongs to a broad category of cell lines, including other kinds of hepatomas [21,23], various categories of leukocytes [56–61], Ehrlich ascites tumour cells [45] and colon cancer [29,62] that undergo apoptosis in response to micromolar concentrations of unsaturated rather than saturated nonesterified fatty acids. Apoptosis in most of these cell types follows the mitochondrial pathway. This also appeared to be the case in the present investigation for AS-30D cells in which cytochrome *c* release, activation of caspase-3 and translocation of the proapoptotic protein Bax from the cytosol to mitochondria were demonstrated.

Because of high affinity of long-chain fatty acids to serum albumin [46], in the present investigation, the cells were exposed to the action of fatty acids when suspended in PBS without foetal serum and only after 30 min the complete incubation medium was added.

According to our present understanding, three events in the mitochondrial metabolism, elicited by fatty acids, could be considered as potential primary causes of the programmed cell death: (i) disruption of energy coupling due to the protonophoric effect of fatty acids [1], (ii) promotion of PTP opening [7,8,63], and (iii) ROS generation.

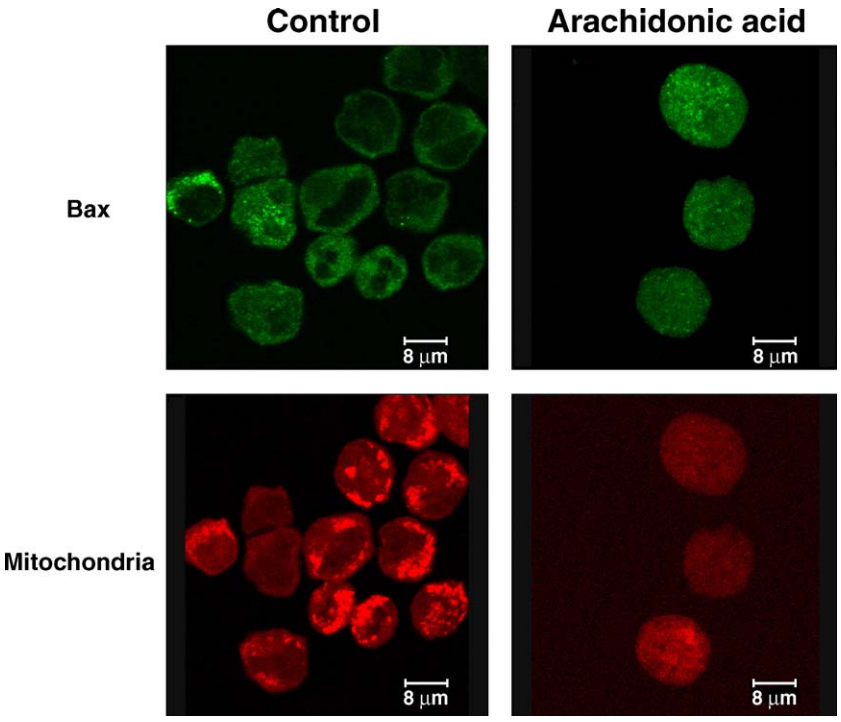

Fig. 10. AS-30D cells stained for mitochondria with MitoTracker CMXRos (red) and Bax (green). The cells were incubated for 24 h without (control) or with arachidonic acid, 60 nmol/mg protein. Punctated distribution of Bax is clearly visible in cells incubated with arachidonic acid, whereas intense staining with MitoTracker disappears.

4.1. Ad (i)

Diminution of ATP production due to mitochondrial uncoupling cannot be the only cause, because, firstly, saturated fatty acids that can also uncouple oxidative phosphorylation, though at higher concentrations, were practically inactive as apoptosis inducers in this cell line; and secondly, chemical protonophores, like CCCP, alone were only slightly proapoptotic.

4.2. Ad (ii)

Opening of PTP could be considered as one of the causative factors, independently of its effect on mitochondrial $\Delta\psi$. Such mechanism has been, for example, proposed for hepatoma MH1C1 cells [64]. Our experiments on cell respiration indicate that, in fact, arachidonic and oleic acids most likely do produce PTP opening in intact AS-30D cells, as their stimulation of O_2 uptake was partly prevented by PTP blocker CsA (Fig. 1). As shown previously by Schönfeld and Bohnensack [7] and ourselves [8,9] in experiments with isolated mitochondria, fatty acids may promote opening of PTP by two mechanisms: (i) an indirect one by partly collapsing $\Delta\psi$ and thus increasing its opening probability [65], and (ii) a direct one by interaction with the pore assembly. PTP opening requires a certain degree of calcium accumulation in mitochondria. This is likely to occur in the present experiments because arachidonic acid has been found to induce elevation of the cytosolic Ca^{2+} concentration, apparently due to a release from the endoplasmic reticulum stores and an influx of Ca^{2+} from the medium (Fig. 4). The RPMI-1640 medium contains 0.4 mM Ca^{2+}. A similar calcium-mobilizing action of arachidonic acid [66] and phytanic acid [67], a branched-chain saturated fatty acid, has also been observed by other authors.

It has to be mentioned that diminution of the respiration rate by higher concentrations of oleic and arachidonic acids, in particular, when followed by FCCP, can also point to PTP opening and the resulting leakage of cytochrome *c* and nicotinamide nucleotides. It has been shown [65] that a collapse of $\Delta\psi$ contributes to PTP opening, and this may be the case when addition of the fatty acid is followed by FCCP.

A clear-cut argument for the participation of PTP in fatty acid-induced apoptosis could be provided by experiments with CsA, as has been done by Scorrano et al. [21] for MH1C1 hepatoma cells where CsA protected against apoptosis induced by arachidonic acid. Such approach was, however, difficult in case of AS-30D cells that appeared to be sensitive to CsA alone. Thus, the involvement of PTP in induction of apoptosis in AS-30D hepatoma by fatty acids can be inferred so far only indirectly.

4.3. Ad (iii)

Relation between apoptosis and ROS is well documented [67–71], although little is known about its molecular mechanism(s). This mechanism of apoptosis induction may be considered for arachidonic acid but not for oleic acid, as the latter one diminished ROS production, most likely because of its uncoupling effect [11]. In contrast, arachidonic acid stimulated ROS generation in AS-30D cells (Fig. 3), the effect that has also been observed in isolated heart mitochondria [10] and has been ascribed to inhibition of complexes I and III of the respiratory chain. Inhibition of excessive ROS production by the strong antioxidant Trolox was paralleled by prevention of arachidonic acid-induced apoptosis (Table 2). In contrast, the protonophore CCCP, which also decreased ROS generation (Fig. 3), had no such protective effect against apoptosis. However, it has to be remembered that protonophores may exhibit a dual effect on apoptosis. On one hand they decrease ROS generation and thus protect against cell death, but on the other hand they may increase opening probability of PTP and thereby promote apoptosis as discussed above.

Collectively, although apoptosis in AS-30D cells elicited by oleic and arachidonic acids proceeds along the mitochondrial pathway, the mechanisms of the cell decay may be different for each acid. Experimental evidence presented in this work clearly points to stimulation of ROS production as the main mechanism of the proapoptotic effect of arachidonic acid. Independently, both acids may induce programmed cell death by triggering the PTP opening, although a direct evidence for this mechanism is lacking so far.

It is noteworthy that in any case the cells were exposed to the action of the fatty acids essentially for a relatively short period of time, namely 30 min, when the cells were incubated with the fatty acid in PBS medium, i.e., before the complete medium containing 10% calf serum was added, resulting in complexing of most of the fatty acid by serum albumin (Fig. 5). Therefore, the two mechanisms discussed above can be considered as signalling agents rather than as chronic factors for the programmed cell death, as it fully developed 24 h after addition of the fatty acid when it was practically no longer present in the cell in the nonesterified form. It cannot be excluded, however, that arachidonic acid was effective not only as free, nonesterified, acid but also in form of its metabolites, e.g., products of cyclooxygenase and lipoxygenase activities [72,73]. In addition, a prolonged incubation of the cells with unsaturated fatty acids may cause enrichment in these fatty acids of their lipids, mainly those of cell membranes, as observed, for example, for human fibroblasts. Such enrichment may result in an increased production of ROS and activation of transcription factors [74].

These results, obtained for a highly malignant hepatoma cell line, may draw attention to unsaturated fatty acids as a potentially beneficial dietary factor in prophylaxis against liver cancer.

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